EXHIBIT B

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DNA Interaction with Naturally Occurring Antioxidant Flavonoids Quercetin, Kaempferol, and Delphinidin

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Abstract

Flavonoids are strong antioxidants that prevent DNA damage. The anticancer and antiviral activities of these natural products are implicated in their mechanism of actions. However, there has been no information on the interactions of these antioxidants with individual DNA at molecular level. This study was designed to examine the interaction of quercetin (que), kaempferol (kae), and delphinidin (del) with calf-thymus DNA in aqueous solution at physiological conditions, using constant DNA concentration (6.5 mmol) and various drug/DNA(phosphate) ratios of 1/65 to 1. FTIR and UV-Visible difference spectroscopic methods are used to determine the drug binding sites, the binding constants and the effects of drug complexation on the stability and conformation of DNA duplex.

Structural analysis showed quercetin, kaempferol, and delphinidin bind weakly to adenine, guanine (major groove), and thymine (minor groove) bases, as well as to the backbone phosphate group with overall binding constants $K_{que} = 7.25 \times 10^4 M^{-1}$, $K_{kae} = 3.60 \times 10^4 M^{-1}$, and $K_{del} = 1.66 \times 10^4 M^{-1}$. The stability of adduct formation is in the order of que>kae>del. Delphinidin with a positive charge induces more stabilizing effect on DNA duplex than quercetin and kaempferol. A partial B to A-DNA transition occurs at high drug concentrations.

Key words: DNA, Antioxidant, Flavonoids, Binding sites, Binding constant, Conformation, FTIR, UV-Visible spectroscopy.

Introduction

Flavonoids are ubiquitous in plants and recognized as the pigments responsible for the color of leaves, especially in autumn. They are found in seeds, citrus fruits, olive oil, tea, and red wine. They have low molecular weights organic compounds composed of three-ring structure with various substitutions. The presence of oxy and hydroxyl groups as well as double bonds in specific positions (Scheme 1) make them strong antioxidants. The antioxidant activity of flavonoids is recently reviewed and the applications of these naturally occurring compounds in medicine are discussed (1). Delphinidin inhibits activator protein 1 activity and cell transformation (2). Quercetin and kaempferol inhibit transcription with RNA polymerase II in normal human fibroblasts (3) and exhibit complex interaction with DNA and RNA polymerases (4). A review of clinical applications including anticancer and antiviral activities of quercetin has been recently reported (5). It has also been demonstrated that low flavonoid concentration stabilizes DNA duplex, whereas helix destabilization can occur when DNA incubated for a long time with high flavonoid content (6). Even though much is reported about antioxidant and antitumor antitumor activities of flavonoids (2), little is known about their interactions with individual DNA and RNA. Therefore it was of interest to examine the bindings of these flavonoids with DNA at molecular level.

Abbreviations: que, quercetin; del, delphinidin; kae, kaempferol; FTIR, Fourier transform infrared.

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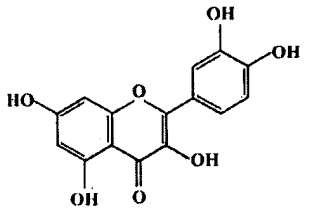
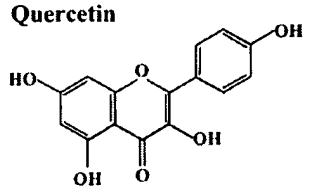
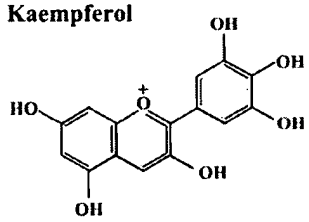
Scheme 1



Quercetin



Kaempferol



Delphinidin

We now report the results of FTIR and UV-Visible spectroscopic analysis of DNA interaction with quercetin, kaempferol, and delphinidin in aqueous solution at physiological conditions, using constant DNA concentration (6.5 mmol) and various drug/DNA(phosphate) ratios of 1/65 to 1. The drug binding site, the binding constant, and the effects of drug complexation on the stability and conformation of DNA duplex are discussed here.

Material and Methods

Materials

Quercetin, delphinidin, and kaempferol were purchased from Extrasynthese (France). Highly polymerized type I calf-thymus DNA sodium salt (7% Na content) was purchased from Sigma Chemical Co., and deproteinated by the addition of $CHCl_3$ and isoamyl alcohol in NaCl solution. The absorbance at 260 and 280 nm was recorded, in order to check the protein content of DNA solution. The A_{260}/A_{280} ratio was 1.85, showing that the DNA was sufficiently free from protein (7). Other chemicals were of reagent grade and used without further purification.

Preparation of Stock Solutions

Sodium-DNA (5 mg/ml) was dissolved in distilled water (pH 7.20) at 5° C for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The final concentration of the calf-thymus DNA solution was determined spectrophotometrically at 260 nm using molar extinction coefficient ε_{260} = 6600 $cm^{-1}M^{-1}$ (expressed as molarity of phosphate groups) (8, 9). The UV absorbance at 260 nm of a diluted solution (1/250) of calf-thymus DNA used in our experiments was 0.661 (path length was 1 cm) and the final concentration of the DNA solution was 25 mM in DNA phosphate. The average length of the DNA molecules, estimated by gel electrophoresis was 9000 base pairs (molecular weight ~ 6×10^6 Da). The appropriate amounts of quercetin, kaempferol and delphinidin (0.3 to 25 mM) were prepared in distilled water-ethanol (50/50%) and added dropwise to DNA solution, in order to attain the desired pigment/DNA(P) molar ratios (r) of 1/65 to 1 with a final DNA concentration of 6.5 mM. The pH of the solutions was adjusted at 7.0 ± 0.2, using NaOH solution.

FTIR Spectra

Infrared spectra were recorded on Magna 750 FT-IR spectrophotometer (DTGS detector, Ni-chrome source and KBr beam splitter) with a total of 100 scans and resolution of 4 cm^{-1}. Spectra were collected and manipulated using the OMNIC (ver. 3.1) software supplied by the manufacturer of the spectrophotometer. Solution spectra were recorded after 1 hour of incubation, using AgBr windows. The water subtraction was carried out using water/ethanol (50/50%) containing 0.1 M NaCl solution at pH = 7.0 ± 0.2 as a reference. A good water subtraction is considered to be achieved if there is a flat baseline around 2200 cm^{-1}, where the water combination mode is located. This method yields a rough estimate of the subtraction scaling factor, but it removes the spectral features of water in a satisfactory manner (10).

The difference spectra [(DNA solution + flavonoid solution) - (DNA solution)] were obtained using a sharp DNA band at 968 cm^{-1} as an internal reference. This band, which is due to deoxyribose C-C and C-O stretching vibrations, exhibits no spectral changes (shifting or intensity variation) upon flavonoid-DNA complexation and cancelled out upon spectral subtraction (11).

The plots of the relative intensity (R) of several peaks of DNA in-plane vibrations related to A-T, G-C base pairs and the PO_2^- stretching vibrations such as 1710 (guanine), 1663 (thymine), 1608 (adenine), 1498 (cytosine), and 1225 cm^{-1}

DNA adducts are coming from drug vibrational frequencies and they are not due to DNA vibrations (Fig. 2, r = 1/65).

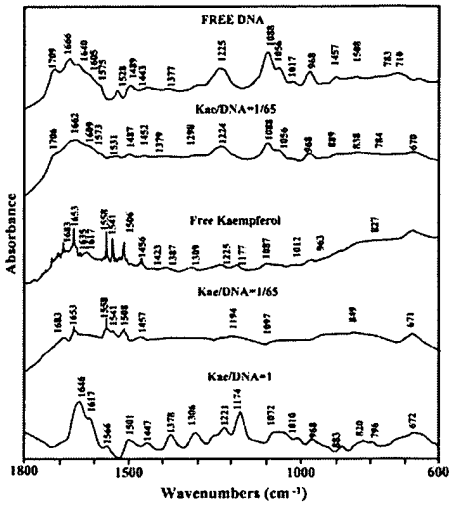


Figure 2: FTIR spectra and difference spectra [(DNA solution + kaempferol solution) - DNA solution)] of the free calf-thymus DNA and kaempferol complexes in aqueous solution.

As kaempferol concentration increased, shifting for the bands at 1710 (guanine) to 1706, 1663 (thymine) to 1662, 1608 (adenine) to 1609, and 1225 (PO_2) to 1224 cm^{-1} was observed in the spectra of drug-DNA complexes (Fig. 2). The observed shifting was accompanied by a major increase in intensity (10%) of these vibrations. The positive features at 1701, 1687, and 1608 cm^{-1} in the difference spectra are due to an increase in intensity of the guanine, thymine, and adenine bands at 1710, 1663, and 1608 cm^{-1}, upon drug complexation (Fig. 2, r = 1). The observed spectral changes are due to indirect interaction of kaempferol (*via* OH groups) with guanine, adenine, and thymine bases. It should be noted a band with medium intensity at 1640 cm^{-1} in the IR spectra of the free DNA and at 1653 and 1646 cm^{-1} in the difference spectra of drug-DNA adducts is due to water deformation mode and it is not coming from DNA vibrations (Fig. 2). The major increase in intensity of DNA vibrations at high kaempferol contents can be also attributed to some degree on helix destabilization. Similar intensity increase was also observed for DNA vibrations when incubated with high quercetin concentration (discussed earlier).

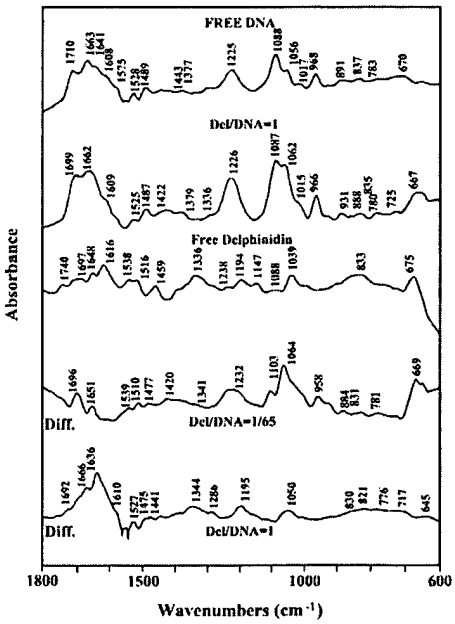


Figure 3: FTIR spectra and difference spectra [(DNA solution + delphinidin solution) - DNA solution)] of the free calf-thymus DNA and delphinidin complexes in aqueous solution.

Delphinidin-DNA Interaction

At low delphinidin concentration (r = 1/65), a major drug interaction with backbone phosphate group was observed. This interaction is through positively charged delphinidin oxy with PO_2 group, which lead to the phosphate charge neutralization and helix stabilization. Evidence for this comes from the shift of the PO_2 band at 1225 to 1226 cm^{-1} with major increase in the intensity of the phosphate band (20%) (Fig. 3, r = 1/65). The presence of a broad peak centered at 1232 and a shoulder band at 1103 cm^{-1} in the difference spectrum of the drug-DNA adduct is due to the increase in intensity of the phosphate bands, as a result of delphinidin-PO_2 interaction (Fig. 3, r = 1/65). The drug-phosphate interaction is mainly through H-bonding between polyphenol OH groups and the backbone PO_2 groups.

As delphinidin concentration increased, shifting for the bands at 1710 (guanine) to 1699, 1663 (thymine) to 1662, and 1608 (adenine) to 1609 cm^{-1} was observed in the spectra of drug-DNA complexes (Fig. 3, r = 1). The observed shifting was accompanied by a major increase in intensity (15%) of these vibrations. The positive features at 1692, 1666, and 1610 cm^{-1} in the difference spectra of delphinidin-DNA adducts are due to an increase in intensity of the guanine, thymine and adenine bands at 1710, 1663, and 1608 cm^{-1}, upon drug complexation (Fig. 3, r = 1). The observed spectral changes are due to indirect interaction of delphinidin (*via* OH

and O^+) with guanine, adenine, and thymine bases. It should be noted that a band with medium intensity at 1640 cm^{-1} in the spectra of the free DNA and at 1651 and 1636 cm^{-1}, in the difference spectra of drug-DNA adducts is due to water deformation mode and it is not arising from DNA vibrations (Fig. 3). The major increase in intensity of DNA vibrations at high delphinidin contents can also be attributed to some extent on helix destabilization. Similar intensity increase was also observed for DNA vibrations when incubated with high cation concentration (17-19).

DNA Conformation

A partial B to A-DNA transition occurred upon flavonoid adduct formation at high drug concentrations. Evidence for this comes from the shift of the sugar-phosphate band at 837 cm^{-1} (B-DNA marker) towards a lower frequency with major reduction in its intensity. In the difference spectra of flavonoid-DNA complexes (r = 1), the emergence of a new peak at about 820 cm^{-1} (A-DNA marker) accompanied by major loss of the intensity of the band at 830 cm^{-1} (B-DNA marker). Similarly, the other B-DNA marker band at 1710 shifted to 1706-1699 upon drug complexation (Figs. 1, 2, and 3, r = 1). However, other B-DNA marker band at 1225 cm^{-1} showed no major shifting in the spectra of flavonoid-DNA adducts (Figs. 1, 2, and 3). In a complete B to A transition, the B-DNA marker bands are observed at 1710-1700 cm^{-1}, 1225-1240 cm^{-1}, 825-800 cm^{-1}, respectively, and a new band appears at about 870-860 cm^{-1} (14-16). The observed shifting for the bands at 837 and 1710 cm^{-1}, is due to a partial reduction of the B-DNA towards A-DNA upon flavonoid complexation.

Stability of flavonoid-DNA adducts

The binding constants K were estimated according to the methods described (20-22). Assuming that there is only one type of interaction between drug and DNA in aqueous solution, the equations 1 and 2 can be established:

$$\text{DNA + drug} \leftrightarrow \text{DNA:drg} \qquad [1]$$

$$K = \frac{[\text{DNA:drug}]}{[\text{DNA}][\text{drug}]} \qquad [2]$$

where K is the binding constant and drug = quercetin (que) or kaempferol (kae) or delphinidin (del).

Assuming [DNA:drug] = C_B,

$$K = \frac{C_B}{(C_B - C_{DNA})(C_B - C_{drug})} \qquad [3]$$

where, C_{DNA} and C_{drug} are the analytical concentration of DNA and drug in solution, respectively.

According to the Beer-Lambert law:

$$C_{DNA} = \frac{A_0}{\varepsilon_{DNA} \cdot l} \qquad [4]$$

$$C_B = \frac{A - A_0}{\varepsilon_B \cdot l} \qquad [5]$$

where, A_0 and A are the absorbance of DNA at 260 nm in the absence and presence of pigment, respectively. ε_{DNA} and ε_B are the molar extinction coefficient of DNA and the bound drug, respectively. l is the light path of the cuvette (1cm).

By displacing ε_{DNA} and ε_B in the Equation 3 by Equations 4 and 5, the Equation 6 can be deduced:

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_{DNA}}{\varepsilon_B} + \frac{\varepsilon_{DNA}}{\varepsilon_B \cdot K} \cdot \frac{1}{C_{drug}} \qquad [6]$$

(PO_2^- groups), versus the polyamine concentrations were obtained after peak normalization using:

$$R_i = \frac{I_i}{I_{968}} \qquad [1]$$

Where I_i is the intensity of absorption peak for pure DNA and DNA in the complex with i concentration of pigment, and I_{968} is the intensity of the 968 cm^{-1} peak (internal reference) (11).

UV-Visible Spectra

The UV spectra are recorded on Jasco V-550 with pigment concentrations of 0.01 to 1 mM and constant DNA concentration of 0.1 mM.

Results and Discussion

Quercetin-DNA Interaction

At low quercetin concentration (r = 1/65), no major drug-DNA interaction was observed. Evidence for this comes from no major shifting or intensity variations of the DNA in-plane vibrations at 1710 (guanine), 1663 (thymine), 1608 (adenine), 1498 (cytosine) and the back bone phosphate group at 1225 (asymmetric PO_2 stretch) and 1088 cm^{-1} (symmetric PO_2 stretch) (10-16). Several positive features at 1718, 1682, 1653, 1451, 1558, 1510, 1458, and 669 cm^{-1} in the difference spectra of drug-DNA adducts at r = 1/65 are coming from quercetin vibrational frequencies and not from DNA vibrations (Fig. 1). However, positive peaks at 1222 and 1061 cm^{-1} are due to an increase in the intensity of the phosphate stretching vibrations as a result of drug-PO_2 interaction (Fig. 1). A minor loss of intensity of the DNA in-plane vibrations at 1710, 1663, 1489 cm^{-1} can be attributed to a partial helix stabilization as a result of drug-phosphate binding. The loss of intensity of DNA vibrations was also attributed to duplex stabilization upon cation-PO_2 interaction (17).

As quercetin concentration increased, shifting for the bands at 1710 (guanine) to 1706, 1663 (thymine) to 1664, and 1608 (adenine) to 1616 cm^{-1} was observed in the spectra of quercetin-DNA complexes (Fig. 1). The observed shifting was accompanied by a major increase in intensity (10%) of these vibrations. The positive features at 1701, 1687, and 1608 cm^{-1} in the difference spectra are due to an increase in intensity of the guanine, thymine, and adenine bands at 1710, 1663, and 1608 cm^{-1}, upon drug complexation (Fig. 1, r = 1). The observed spectral changes are due to indirect interaction of quercetin (*via* OH groups) with guanine, adenine, and thymine bases. It should be noted that the absorption bands with medium intensity at 1640 cm^{-1} in the IR spectra of the free DNA, at 1652 for the complex and at 1653 cm^{-1} in the difference spectra of drug-DNA adducts are due to water deformation mode and they are not coming from DNA vibrations (Fig. 1). The major increase in intensity of DNA vibrations at high quercetin contents can be also attributed to some degree of helix destabilization. Similar intensity increase was also observed for DNA vibrations in the presence of high copper concentration (17). Evidence for helix destabilization was also observed when DNA incubated with high flavonoid concentrations (6).

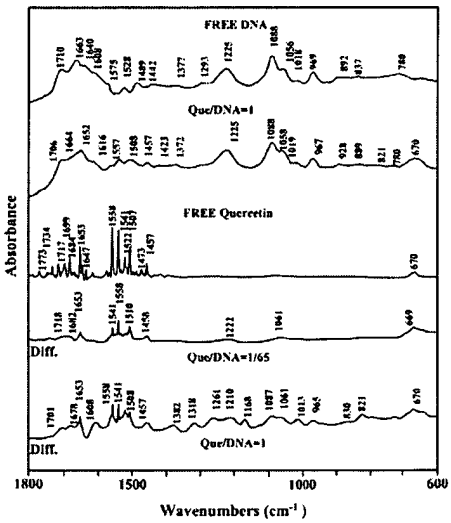


Figure 1: FTIR spectra and difference spectra [(DNA solution + quercetin solution) - DNA solution)] of the free calf-thymus DNA and quercetin complexes in aqueous solution.

Kaempferol-DNA Interaction

No major drug-DNA interaction was observed at low kaempferol concentration (r = 1/65). Evidence for this comes from no major spectral variations of the DNA in-plane vibrations at 1710 (guanine), 1663 (thymine), 1608 (adenine), 1498 (cytosine), and the back bone phosphate bands at 1225 (asymmetric PO_2 stretch) and 1088 cm^{-1} (symmetric PO_2 stretch) (Fig. 2). Several positive peaks at 1683, 1558, 1541, 1508, 1457, 1194, and 1097 cm^{-1}, in the difference spectra of kaempferol-

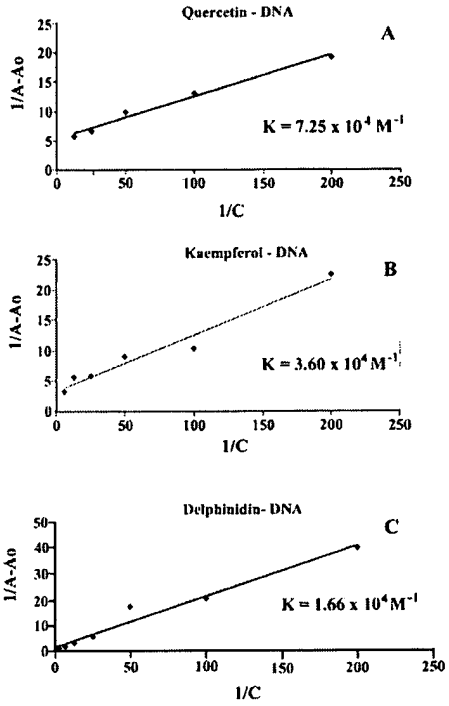


Figure 4: The plot of 1/(A0-A) vs. 1/C for DNA and its drug complexes where A0 is the initial absorption of DNA (260 nm) and A is the recorded absorption at different drug concentrations (C).

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Thus, the double reciprocal plot of

$$\frac{1}{A - A_0} \text{ vs. } \frac{1}{C_{drug}} \text{ (or } \frac{1}{[drug]}\text{)}$$

is linear and the binding constants (K) can be estimated from the ratio of the intercept to the slope (17-19). Our data of 1/[drug complexed] almost proportionally increased as a function of 1/[free drug] (Fig. 4). The overall binding constants estimated were $K_{que} = 7.25 \times 10^4 M^{-1}$, $K_{kae} = 3.60 \times 10^4 M^{-1}$, and $K_{del} = 1.66 \times 10^4 M^{-1}$ (Fig. 4). The overall binding affinities observed indicate of a weak drug-DNA interaction. The lower K values obtained for delphinidine over quercetin and kaempferol can be attributed to the presence of a positive charge associated with delphinidin, which makes del-DNA interaction largely electrostatic. It should be noted that even the estimated K values are small, major DNA structural changes occurred upon flavonoid interaction.

Summary

On the basis of our spectroscopic results the following remarks can be made: (i) The low flavonoid concentrations induce duplex stabilization, while higher drug contents cause helix destabilization; (ii) No major drug-base binding occurs at low flavonoid concentrations, while at high drug contents both base and phosphate interactions are observed; (iii) The order of the stability of adduct formation is quer>kae>del and (iv) A partial B to A-DNA transition occurs at high drug contents.

Acknowledgements

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